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14. ABSTRACT
Our studies to date have determined that human oogonial stem cells, while far less stable than their murine counterparts, can be successfully expanded and propagated in culture. This has allowed us to begin testing the potential tumorigenicity of these cells with the ultimate goal of comparing the DNA signature of the oogonial stem cell-derived tumors to that of primary human ovarian cancer. We have also successfully introduced in human oogonial stem cells genetic alterations commonly detected in ovarian cancer. We are now generating tumors from these altered oogonial stem cells and will compare the histologic features in the tumors formed from the modified oogonial stem cells to those of primary tumors collected from women diagnosed with serous ovarian cancer. Also of importance is the identification of Ddx4-positive cells in xenografts derived from primary human ovarian serous tumors. The biological significance of this apparent rare population is yet to be determined. We have designed and implemented initial studies to test the relative tumorigenicity of Ddx4-positive, CD133-positive and Ddx4 CD133 double positive fractions. Demonstrating that these highly specialized human oogonial stem cells have the capacity to form ovarian tumors would be a major paradigm shift.

15. SUBJECT TERMS
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INTRODUCTION:

High grade ovarian cancer remains one of the most deadly gynecologic cancers. The high mortality rate is often attributed to our inability to detect early stage disease, the high incidence of recurrent chemoresistant disease and our limited understanding of the biology underlying early events in ovarian cancer development as reflected in continued debate surrounding the cell and site of origin of ovarian cancer. Our preliminary data generated using oogonial stem cells derived from the mouse ovary suggested that these rare cells have the capacity to form tumors in immunocompromised mice when 'misplaced' outside of the ovary. Specifically, while no tumor developed when oogonial stem cells were injected directly into the ovary, injection of the same cells into the mouse abdominal cavity resulted in tumors that histologically resemble ovarian cancer. Subsequent studies have identified the presence of a similarly rare oogonial stem cell population in the human ovary. We hypothesize that human oogonial stem cells either shed from the ovarian surface during ovulation or naturally present in an aged ovary have tumorigenic potential. Furthermore, we believe a genetic misprint will allow these cells to undergo additional changes common to the different ovarian cancer subtypes. Our objectives, therefore, were to determine the tumorigenic capacity of human oogonial stem cells and compare the DNA signature of the oogonial stem cell-derived tumors to that of primary human ovarian cancer. In addition, our objective was to induce in human oogonial stem cells genetic alterations commonly detected in ovarian cancer and compare the histologic features in the tumors formed from the modified oogonial stem cells to those of primary tumors collected from women diagnosed with serous ovarian cancer. Demonstrating that these highly specialized human oogonial stem cells have the capacity to form ovarian tumors would be a major paradigm shift.

Recently, the idea that these OSCs can be isolated by the cell surface marker DDX, also known as VASA, has become controversial. In 2012 Tilly et al. reported DDX4 had a carboxyl terminal domain that was expressed on the extracellular membrane and could be used to isolate OSCs. More recent data has challenged this finding. In addition, our own validation studies led us to question the surface expression of DDX4. Our original DOD proposal was predicated on the fact that these rare OSCs expressed DDX4 on the surface and DDX4 expression could be validated by semi-quantitative PCR using methods published by Tilly and colleagues. Given the recent controversy, we conducted additional validation experiments using more extensive methodology. We have completed these studies which revealed some very interesting findings detailed below. While effort on our original aims was suspended until we could validate the identity of the cells we were working with, we now have resumed our original objective which is to assess the tumorigenic capacity of the OSCs. Consequently, we requested and were granted a no cost extension to complete our objectives.

BODY:

Task 1: Tumorigenic assessment of OSCs isolated from reproductive age and menopausal women.

Subtask 1a: In Aim 1 human OSCs isolated from a total of 10 patients (5 reproductive age

women and 5 peri-/post-menopausal women) will be expanded in vitro under conditions established in our laboratory.

As mentioned in the previous report, both the ovarian samples and oogonial stem cell preparations/cultures isolated from pre- and post-menopausal ovaries obtained prior to receiving this funding did not survive long-term cryopreservation. We therefore modified our cryopreservation methods to ensure viability of both the ovarian samples and more importantly, the isolated oogonial stem cell cultures. Following optimization, we actively worked with MGH GYN Tissue Repository staff to accrue new samples and to restock the ovarian tissue required for the proposed studies. Since receiving the award, we have acquired, via the MGH GYN Tissue Repository, human ovarian tissue from nine different patients who fit the relevant clinical criteria. A subset of these ovarian samples has been processed to generate oogonial stem cell cultures. The remainder has been cryopreserved for future isolation of oogonial stem cells. The collected samples comprise ovarian tissue from pre- and post-menopausal women. As expected, the majority of these samples are from post-menopausal women. We will continue to accrue samples in order to obtain sufficient numbers of ovaries from pre-menopausal women.

Three of the newly acquired ovarian samples were subjected to cell sorting to generate DDX4-positive oogonial stem cells. Cells obtained from two of the three sorted ovarian samples were healthy and could be successfully passaged and expanded for validation studies (Figure 1). In RT-PCR analyses, primers corresponding to *PRDM1*, *DPPA3*, *IFITM3*, *TERT*, *DDX4* and *beta – actin* as published by Tilly et al were used to confirm the oogonial stem cell profile of the cultured cells. Relevant controls included oocyte preparations, whole ovary tissue, testicular tissue (positive control) and fibroblasts (negative control).

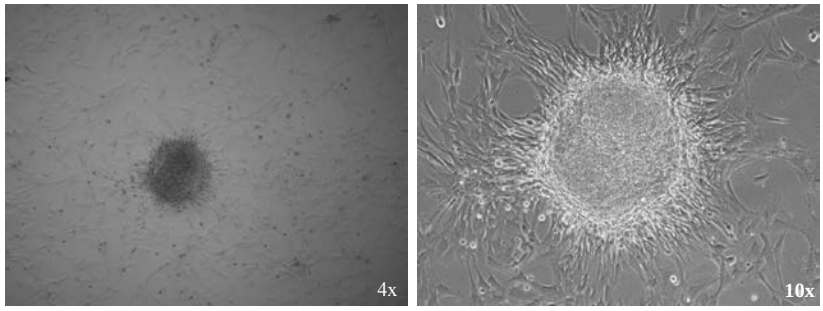

Figure 1. *OSC colony formation following ex vivo expansion.* DDX4+ OSCs isolated from an ovarian sample obtained from a 57 year old post-menopausal patient were expanded in culture. Shown here are images of a colony that formed 17 days after sorting. Left panel, 4x; right panel, 10x.

Recent controversy over the isolation of OSCs via expression of the cell surface marker DDX4 (also known as VASA) is due in part to the historic consideration of DDX4 as an exclusively intracellular protein. In 2012 Tilly et al. reported DDX4 had a carboxyl terminal domain that was expressed on the extracellular membrane and could be used to isolate OSCs. Although current evidence supports both classifications of DDX4, our original DOD proposal was built on the assumption that these rare cells of interest expressed DDX4 on the surface and this expression could be validated by PCR using the methods published by Tilly and colleagues. Given the recent controversy, we sought to conduct additional validation steps using more extensive methodology.

Flow cytometric analysis and sorting

To further characterize the cellular expression of DDX4, we subjected fresh or fresh frozen human and mouse ovarian tissue to cell sorting to generate DDX4 positive and DDX4 negative populations using the antibody and sorting protocol published in White et al. The relative frequency of DDX4 antibody positive cells was highly variable with DDX4 antibody positive cells comprising 4.5-24% of human ovarian cells and 1.9-3.7% of mouse ovarian cells. Surprisingly, isolated antibody negative cells became highly antibody positive in culture (Figure 2). Furthermore, antibody positive, antibody negative and unsorted cells expressed similar levels of antibody positivity over sequential passages (Figure 2).

Expression of DDX4

As observed in the mouse, the level of *DDX4* expression in the human testicle is overwhelming when compared to expression in the ovary. Low *DDX4* expression was detected in the ovary derived from a younger woman (38 years of age) with no expression detected in the ovary of an older woman (57 years of age) consistent with the lack of oocytes observed on histology. Freshly isolated ovarian DDX4 antibody positive and negative cells were assessed by qPCR and compared to the unsorted populations. Neither population showed evidence of *DDX4* expression when compared to the positive control. Again mRNA expression levels of *PRDM1, IFITM3,* and *DPPA3,* were assessed immediately after sorting and again after culturing. With the exception of *PRDM1,* no clear pattern or differences were observed in *IFITM3,* and *DPPA3* among the sorted or cultured antibody positive and antibody negative cells.

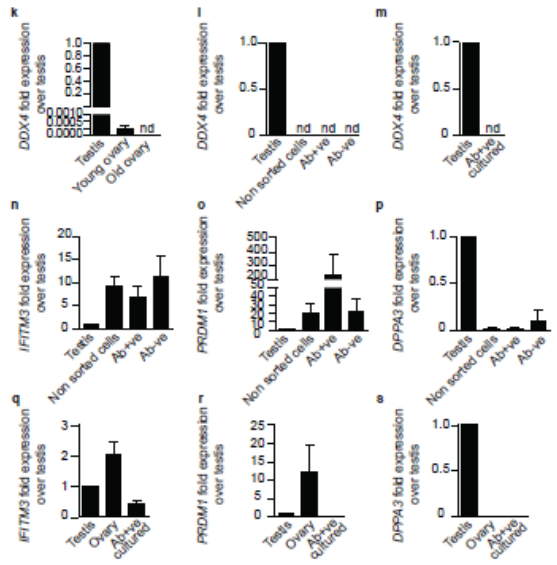

Figure 2. *DDX4* was detected in young human ovaries and in testis, but not in ovaries from old women by qPCR (k). No *DDX4* was detected by qPCR in non sorted, DDX4 Ab+ve and Ab-ve human ovarian cells (l). Similarly, DDX4 Ab+ve isolated and cultured ovarian cells did not express *DDX4* by qPCR compared to testis (m). Freshly isolated non sorted, DDX4 Ab+ve and DDX4 Ab-ve ovarian cells were assessed by qPCR for *IFITM3*, *PRDM1*, *DPPA3* (n,o,p). Cultured ovarian human DDX4 Ab+ve cells were assessed by qPCR for *IFITM3*, *PRDM1*, *DPPA3* compared to ovary and testis (q,r,s)

Subtask 1b: The cells will be virally transduced to stably express luciferase, and then injected into the intraperitoneal cavity of immunocompromised (NOD/SCID) mice. The injections will be done in a dilution series, ($1x10^5$, $1x10^4$, $1x10^3$, $1x10^2$ and $1x10^1$ cells, 2 mice per patient per dilution for a total of 100 mice). Cells will also be injected ($1x10^5$) directly into the ovaries of NOD/SCID mice (2 mice per patient for a total of 20 mice) using a 10 μL NanoFil syringe and a beveled 35gauge needle.

We generated a retrovirus that expresses both GFP and luciferase and have recently infected

pools of oogonial stem cells. We now plan to thaw these cells and expand them to sufficient numbers for cell sorting to isolate GFP-positive infected cells. These cells will be re-plated for expansion in culture and the expanded cells will then be introduced into immunocompromised NOD/SCID mice by intraperitoneal injection as described in Subtask 1b. This strategy will also allow the completion of Subtasks 1c-1e.

Subtask 1c: Tumor development, growth and metastatic potential will be evaluated by the Mouse Imaging Program (MGH/Harvard core facility). The rate of tumorigenesis as well as metastatic spread will be assessed. When tumors have reached a sufficient size for evaluation, mice will be sacrificed and tumors harvested. Each tumor will be divided into 3 equal pieces, one will be preserved in paraformaldehyde and processed for histological analysis, one snap-frozen for genomic analysis, and one vitrified and stored in liquid nitrogen for further analysis at a later date.

This subtask has not been initiated.

Subtask 1d: For histological analysis as well as immunohistochemistry, PFA-fixed tumors will be embedded in paraffin, sectioned, placed onto slides and 5 sections per tumor will be stained with hemotoxylin and eosin for phenotypic analysis. Additionally, sections will be analyzed for p53, cytokeratin (CK) 7, CK20, PAX-8, WT-1, and ER by immunohistochemistry using commercially available antibodies.

This subtask has not been initiated.

Subtask 1e: Genomic Assessment of Tumors. Gene array analysis will be conducted on Affymetrix gene chips by our institutional core facility. The resulting information will be compared to published data sets.

SAMPLE	PRIMARY SITE	HISTOLOGY	GRADE	DDX4-CD133-	DDX4+CD133-	DDX4-CD133+	DDX4+CD133+
OVCA1	Peritoneum	Serous	3	83.27	7.43	8.89	0.41
OVCA2	Peritoneum	Serous	3	73.1	10.6	9.04	7.26
OVCA3	Peritoneum	Serous	3	92.7	6.41	0.77	0.078
OVCA4	Ovary	Serous	3	69.1	26.1	3.69	1.05
OVCA5	Peritoneum	Serous	3	92.7	3.02	4.08	0.14
OVCA6	Peritoneum	Serous	3	86.9	11.9	0.71	0.41

Table 1. Flow cytometric analysis of DDX4 and CD133 expression in human ovarian cancer xenografts. The relative frequency (indicated as percent of the total viable population) of cells with the indicated DDX4 and CD133 expression phenotypes was determined in xenografts derived from six independent primary ovarian serous cancers. Note the consistent presence of a DDX4+CD133- population in all analyzed samples.

We also proposed to compare the genomic profile of the tumors generated from the oogonial stem cells with the profile of xenografts derived from primary human serous ovarian cancer and/or primary human serous peritoneal cancer. Toward this goal, we have generated xenografts from such primary tumors which have been harvested and frozen for future nucleic acid isolation.

We previously reported that in parallel analyses, we used flow cytometry to determine the relative frequency of DDX4-positive cells in a subset of xenograft tumors derived from primary human ovarian serous cancer. We detected a DDX4-positive population within every analyzed tumor (Table 1). The presence of this rare sub-population within human ovarian tumors was not reported at that time and its biological significance remains to be determined.

	5×10^4	5×10^3	5×10^2
Bulk	4	4	4
DDX4-CD133-	4	4	4
DDX4+CD133-	4	4	4
DDX4-CD133+	2	6	4
DDX4+CD133+	1	3	8

Table 2. *Assessing the relative tumorigenicity of the isolated cell populations.* Immunocompromised NOD/SCID mice were injected with the indicated cell numbers of each purified cell population in a limiting dilution assay. The numbers in each column represent the total number of mice injected with each cell number. The information reported here comprises three separate injection series using cells derived from three independent patient xenografts. The bulk population represents unsorted tumor cells.

We initiated experiments to test the relative tumorigenicity of tumor-derived DDX4+ cells. Specifically, we isolated purified populations of DDX4+CD133-, DDX4-CD133+, DDX4+CD133+ and DDX4-CD133- cells and have injected them into NOD/SCID mice in a limiting dilution assay (Table 2). Tumor formation was monitored in the injected animals. While there initially appeared to be differences in tumor formation following injection of cells with specific DDX4 and CD133 expression profiles, no consistent difference in the onset of tumor formation as a result of the injection of the different cell populations (DDX4+CD133-, DDX4-CD133+, DDX4+ CD133+ and DDX4-CD133- cells) was evident at the end of the experiment. Interestingly, other investigators have used immunohistochemistry to show that DDX4 is over expressed in a significant number of epithelial ovarian cancers and its expression was positively correlated with advancing age and serous histology. DDX4 positive staining was also observed in ovarian inclusions cysts with metaplastic changes, but absent in cysts without metaplastic changes. More recently published data suggests that DDX4 is co-expressed with the cancer stem cell marker CD133 in ovarian cancer tissue samples and cell lines whereas CD133 negative ovarian cancer cells do not express DDX4. This study is similar to what we had proposed and were actively testing when the above manuscript was published online. Given that the reported results were different than what we were discovering and the report lacked validation of true DDX4 expression, we sought to verify their findings as well as our own.

Characterization of DDX4 in ovarian cancer and established ovarian cancer cell lines

As mentioned, it was previously shown that DDX4 is expressed in ovarian carcinomas and its expression is associated with age and the serous histophenotype. Thus, we analyzed a subset of patient derived xenografts (PDXs) established from primary human ovarian cancer samples. FACS analysis of these tumors revealed a significant percentage (5-20%, Figure 3a) of DDX4 positive cells that were easily isolated. These purified cells, however, showed no evidence of DDX4 expression by qPCR, Western blot and mass spectrometry (not shown).

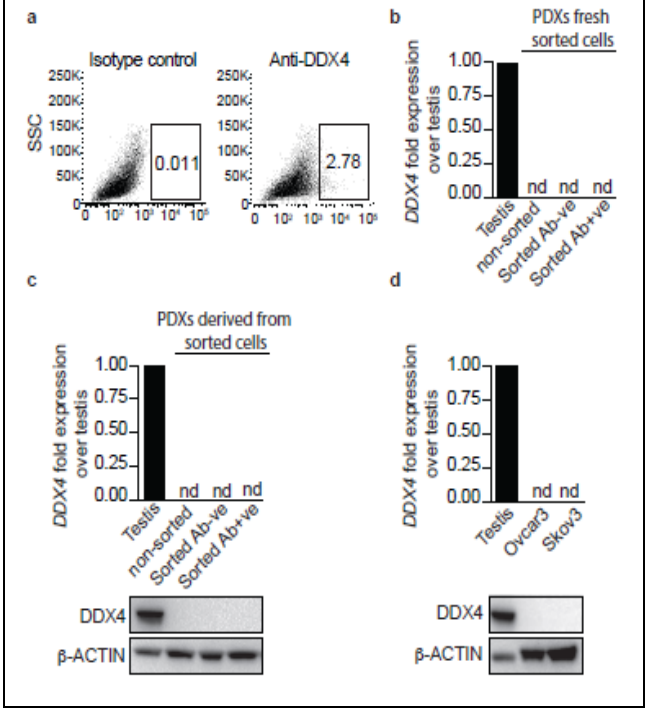

Figure 3. Characterization of DDX4 in ovarian cancer and ovarian cancer cell lines. (a) DDX4 Ab+ve cells were isolated from xenografts (PDXs). (b) Non-sorted, sorted Ab+ve and Ab-ve cells were subjected to qPCR with no evidence of *DDX4* expression compared to the positive control, human testis (nd= not detected) (c) Non sorted and sorted cells were re-injected into mice to generate tumors and again, DDX4 was not detected by qPCR or immunoblot compared to the positive control (n=5-8 mice per group). (d) DDX4 expression was not detected in OVCAR3 and SKOV3 cell lines by qPCR and immunoblot compared to the positive control.

Freshly isolated ovarian tumor antibody positive and negative cells also did not express DDX4 as assessed by qPCR (Figure 3b). Non-sorted, antibody negative and antibody positive cells were injected into NOD/SCID mice. Tumors were harvested after 2 months and analyzed by qPCR and western blot, with no evident DDX4 expression (Figure 3c). Mass spectrometry revealed no positivity for DDX4 in the tumor cells (negative data not shown).

As discussed above, a recent publication provided some evidence to suggest that specific ovarian cancer cell lines expressed significant levels of DDX4 by protein analysis. To this end we assessed the expression of *DDX4* mRNA and protein in the OVCAR3 and SKOV3 established ovarian cancer cell lines. Consistent with the results obtained in human ovarian cells from our PDX model, we detected no expression of either *DDX4* mRNA or protein in OVCAR3 or SKOV3 cells (Figure 3d).

To further test DDX4 protein expression in antibody negative and antibody positive samples, a mass spectrometry approach was utilized. Rhesus testis was used as a positive control to ensure that DDX4 protein could be recognized by mass spectrometry. Next, we analyzed antibody positive cells from murine and human ovaries. In addition to analysis of our benign primary ovarian cells, we also analyzed cells derived from primary ovarian cancer xenografts (PDXs). We detected no DDX4 peptides in any of these murine or human ovarian samples despite easy detection of DDX4 peptides in Rhesus testis.

Task 2: Assessment of induced genetic mutations in human OSCs. Human OSCs will be expanded in vitro (see Subtask 1a). These cells will be retrovirally transformed to stably express either genetic mutations or constitutively active oncogenes. The genetically altered cell lines will be injected into immunocompromised mice and evaluated for changes in tumorigenic characteristics.

Subtask 2a: Retroviral transduction of human OSCs. OSCs transformed with luciferase in Subtask 1b will be stably transduced using the pBABE retroviral vectors already produced in our laboratory (dominant negative TP53, KRAS mutant, constitutively active PIK3CA and AKT1, and control vector; see preliminary data) using the PLAT-A packaging cell line.

Retroviral vectors expressing oncogenic alleles of human *TP53*, *AKT1*, *KRAS*, and *PIK3CA* were constructed and initially validated in both a human endometrial cancer cell line and mouse oogonial stem cells. The expected effect on the expression of relevant genes/proteins was confirmed by RT-PCR or immunoblotting. The viruses were then used to infect a human oogonial stem cell line. Cells infected with the *AKT1*, *KRAS* or *PIK3CA* viruses were generated by puromycin selection five days post-infection and then maintained in a low concentration of puromycin to ensure a stable pure population of virus-containing cells. Since the p53-expressing retrovirus construct does not confer puromycin resistance, successful infection with this virus was confirmed by RT-PCR using primers designed to specifically detect exogenous *TP53* gene expression.

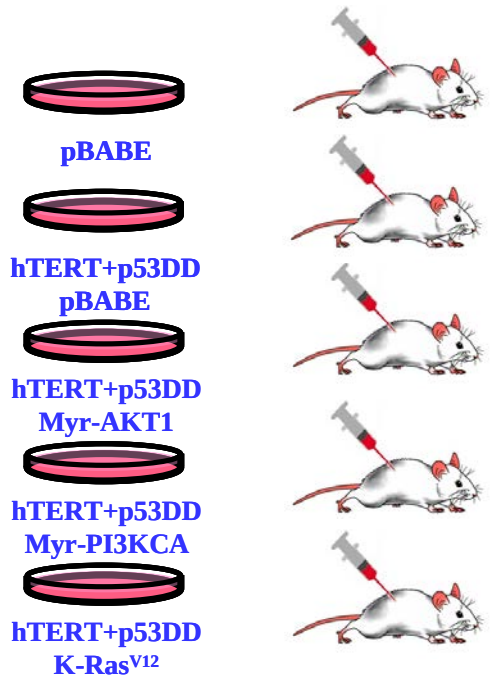

Figure 4. *OSC injection into NOD/SCID mice.* OSC expressing dominant negative p53 either alone or in combination with activated Akt, PI3K or K-Ras were injected into NOD/SCID mice in a limiting dilution assay. OSC cells infected with parent virus alone (pBABE) were also injected. Tumor formation in the injected mice is currently being monitored.

Although co-infection of human oogonial stem cells with the oncogenic *TP53* and *KRAS* retroviruses was highly efficient, co-infection of the *TP53* virus with either the oncogenic *AKT1* or *PIK3CA* viruses was unsuccessful. We determined that initial infection with the TP53 retrovirus followed by subsequent re-infection with the *KRAS*, *AKT* or *PIK3CA* retrovirus was required to generate human oogonial cells that co-expressed the relevant oncogenes as validated by either RT-PCR or immunoblotting.

Subtask 2b: The transformed cell lines (5 lines) will be injected into the intraperitoneal cavity of immunocompromised (NOD/SCID) mice. The injections will be done in a dilution series, ($1x10^5$, $1x10^4$, $1x10^3$, $1x10^2$ and $1x10^1$, 3 mice per mutation, for a total of 60 mice).

We previously reported that we conducted simple preliminary studies to assess the potential impact of the retroviral infections on human oogonial stem cell proliferation. Surprisingly, human oogonial stem cells expressing exogenous oncogenic p53, KRAS, AKT1 or PI3K reached confluency in culture 3-fold faster than cells infected with the control retrovirus. We have since subcutaneously injected varying cell numbers (1×10^6, 1×10^5, 1×10^4, and 1×10^3) of human oogonial stem cells infected with the various retroviruses into NOD/SCID to pre-assess their ability to generate tumors in mice (Figure 4). This will inform our choice of the post-injection time points at which we will assess tumor formation in mice using the more expensive IVIS imaging system. Concurrently, we have introduced a retrovirus which co-expresses GFP and luciferase into the human oogonial stem cell lines expressing the various oncogenes. Following infection, the cells will be sorted based on GFP expression to generate pure populations of infected cells. Those cells will subsequently be injected into the intraperitoneal cavity of NOD/SCID mice and tracked *in vivo* via IVIS imaging of luciferase activity. We had initiated this task but suspended further work once we realized that the isolation of the OSCs via the DDX4 antibody may be not as specific and efficient as previously believed. More specifically, we determined that although the DDX4 antibody could be used isolate a small population of cells that could give rise to oocytes, the cells were not truly DDX4 positive. That is, the DDX4 antibody was indirectly enriching for OSCs but the resulting populations were not pure. Therefore, we delayed further experiments until we could confirm that these cells could give rise to tumors in our corresponding mouse model. We have determined that modified mouse OSCs can generate tumors following injection (Figure 5), albeit with poor efficiency.

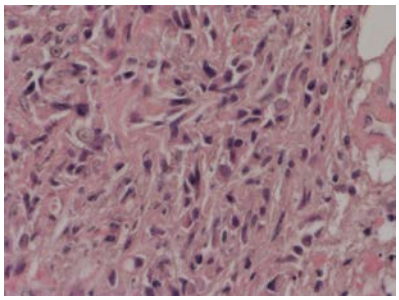

Figure 5. *Histological evidence of tumor formation.* Given the previous challenges we wanted to verify that the OSCs in culture could induce tumor an aliquot of the cells were injected into immunocompromised mice. Over time a poorly differentiated tumor with adenocarcinoma features formed.

Subtask 2d: Tumor development, growth and metastatic potential will be evaluated by the Mouse Imaging Program (MIP, MGH/Harvard core facility). A ll mice will assessed weekly in the IVIS imaging system (each run takes approximately 20-30 minutes). The rate of tumorigenesis as well as metastatic spread will be assessed by the core facility software. When tumors have reached a sufficient size for evaluation, mice will be sacrificed and tumors harvested. Each tumor will be divided into 3 equal pieces, one will be preserved in paraformaldehyde and processed for histological analysis (including immunohistochemistry), one snap-frozen for genomic analysis, and one vitrified and stored in liquid nitrogen for further analysis at a later date.

This subtask has not been initiated

Subtask 2e: As in task 1, tumors will be subjected to histological analysis as well as immunohistochemistry. PFA-fixed tumors will be embedded in paraffin, sectioned, placed onto slides and 5 sections per tumor will be stained with hemotoxylin and eosin for phenotypic analysis. Additionally, sections will be analyzed for p53 signature, CK7, CK20,

PAX-8, WT-1, and ER by immunohistochemistry using commercially available antibodies.

This subtask has not been initiated.

KEY RESEARCH ACCOMPLISHMENTS:

1. The oogonial stem cell preparations/cultures isolated from both pre- and post-menopausal ovaries collected prior to receiving funding did not survive long term cryopreservation. Consequently, we have been restocking our resource of ovarian tissue.

2. We acquired human ovarian tissue from 11 different patients via the MGH GYN Tissue Repository.

3. A subset of these has been cryopreserved for future isolation of oogonial stem cells.

4. It was ascertained that the samples collected represent ovarian tissue from women considered to be pre- and post-menopausal. As expected, the majority of the samples are post-menopausal ovaries.

5. Newly collected samples were subjected to flow cytometric sorting. Only some of the sorted samples yielded viable cells.

6. The viable oogonial stem cell cultures have been successfully passaged and expanded for validation studies.

7. We originally validated the oogonial stem cell nature of the isolated cultures, by using previously published primers for *PRDM1*, *DPPA3*, *IFITM3*, *TERT*, *DDX4* and *beta – actin* in RT-PCR analyses. Relevant controls included oocyte preparations, whole ovary tissue, testicular tissue (positive control) and fibroblasts (negative control). We since incorporated more specific primer sets for qPCR as opposed to RT-PCR, and performed additional methodologies such as mass spectrometry, Image Stream and RNAseq. Taken together, our results showed that the cells of interest do not express DDX4 but can be isolated by the anti-DDX4antibody. This observation suggests that indirect binding of the antibody to the surface will enrich for these cells. The efficiency at which this occurs is not yet known though it is expected to be low.

8. Retroviral vectors expressing oncogenic alleles of human *TP53*, *AKT1*, *KRAS*, or *PIK3CA* were successfully constructed.

9. The viral constructs were initially validated in a human endometrial cancer cell line and mouse oogonial stem cells. The expected effect of each on expression of appropriate genes/proteins was confirmed by RT-PCR or immunoblot analysis.

10. Once validated, the viruses were used to infect a human oogonial stem cell line. Cells infected with the *AKT1*, *KRAS* or *PIK3CA* viruses were generated by puromycin selection

five days post-infection and then maintained in a low concentration of puromycin to ensure a stable pure population of virus-containing cells. As described, the expected effect on the expression of relevant genes/proteins was confirmed by RT-PCR or immunoblotting.

11. Since the p53-expressing retrovirus construct does not confer puromycin resistance, successful infection with this virus was confirmed by RT-PCR using primers designed to specifically detect exogenous *TP53* gene expression.

12. Simple preliminary studies assessing the potential impact of exogenous expression of oncogenic alleles of human *TP53*, *KRAS*, *AKT1* and *PIK3CA* have determined that human oogonial stem cells expressing any of these proteins reach confluency in culture 3-fold faster than cells infected with the control retrovirus.

    **** Based on the validation findings we temporarily suspended our tumorigenicity studies until we could validate the nature of the cells isoalted iwth the anti-DDX4 antibody. We also wanted to repeat the correlative mouse OSC studies (not funded by this award) until we confirmed we could repeat what we observed in our earlier studies. We have determined that mOSCs could indeed induce tumor formation although our more recent experiments required injection of higher numbers of cells expressing oncogenic alleles of *TP53* and *AKT1* to generate tumors at a much lower frequency.

13. In order to compare the genomic profile of tumors generated from human oogonial stem cells to the profile of xenografts derived from human primary serous ovarian or peritoneal tumors, we generated xenografts which have been harvested and frozen for future nucleic acid isolation. Despite previous reports, our preliminary findings suggest that our ovarian tumors samples do not express DDX4 as evidenced by qPCR, western blot and mass spectrometry analyses.

14. Using flow cytometry, we reported that we detected a population of DDX4-positive cells in xenografts derived from primary human serous ovarian and peritoneal tumors. Others have recently reported that this rare sub-population overlaps with a CD133 fraction. We have since disproven this using more extensive validation assays. This work is currently under review.

REPORTABLE OUTCOMES:

- *manuscripts, abstracts, or presentations*

  A manuscript containing our most recent findings regarding DDX4, OSCs and ovarian cancer cells is under review at Nature Medicine.

- *licenses applied for and/or issued;*

  No licenses have been applied for or issued

- *degrees obtained that are supported by this award;*

N/A

- *development of cell lines, tissue or serum repositories;*

  As part of the proposal we have collected samples of ovaries and developed short term cell lines from the oogonial stem cells. Due to IRB restrictions these are not available for distribution.

- *informatics such as databases and animal models, etc.;*

  N/A at this point in time

- *funding applied for based on work supported by this award;*

  N/A

- *employment or research opportunities applied for and/or received based on experience/training supported by this award*

  N/A

CONCLUSION:

By utilizing more extensive validation methods, we have determined that the finding that DDX4 is expressed both intracellularly and extracellularly may not be entirely correct. This discovery led to a temporary hold on our progress related to the tumorigenic potential of OSCs until we could confirm the cells we had isolated for these studies were the same as those previously described by White et al. In a collaborative effort with Dr. Erin Wolff (NIH based scientist), we re-characterized both freshly isolated and cultured OSCs that were purified using the anti-DDX4 antibody. In short, our recent submission to Nature Medicine summarizes our findings from RNA sequencing and mass spectrometry data as well as PCR and immunoblot analysis to provide evidence suggesting that DDX4 antibody positive cells isolated from the ovary and the ovarian tumor samples by FACS had no evidence of DDX4 expression. Moreover, even if DDX4 is over expressed in ovarian stem cells, our results suggest that DDX4 cannot be expressed at the cell surface. It is important to note that Dr. Wolff did provide evidence that these cells could generate oocyte like structures confirming the original findings by White et al. Thus, these data suggest that the antibody is non-specifically binding something novel and this may provide the basis for the indirect identification of stem populations such as OSCs. Given the lack of antibody specificity and low efficiency of isolating true OSCs, the human DDX4-positive sorted cell populations likely only contain a subset of the true cells of interest. We will therefore likely need to increase the number of cells injected into immunocompromised mice in order to test our original hypothesis that OSCs have the potential to become malignant and take on a phenotype similar to that of ovarian cancer as was observed with murine OSCs. We are now able to resume our efforts to assess both the biological significance of this rare population of cells and their tumorigenic potential. We have thawed our original samples and are in the process of generating sufficient cell numbers for injection. ****As mentioned, a subset of this

work was submitted to Nature Medicine. Upon submission, we were asked to merge two independent papers and resubmit. We are now awaiting the outcome of the review process.

REFERENCES:

N/A

APPENDICES:

N/A

SUPPORTING DATA:

Figures have been included within the text.